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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

2′-Hydroxychalcones as an alternative treatment for trichomoniasis in association with metronidazole

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Abstract

The treatment for trichomoniasis, based on 5′-nitroimidazol agents, has been presenting failures related to allergic reactions, side effects, and the emergence of resistant isolates. There are no alternative drugs approved for the treatment of these cases; thus, the search for new active molecules is necessary. In this scenario, chalcones have been extensively studied for their promising biological activities. Here, we presented the synthesis of three hydroxychalcones (**3a**, **b**, and **c**), in vitro and in silico analyses against *Trichomonas vaginalis*. The in vitro biological evaluation showed that hydroxychalcone **3c** presented anti-*T. vaginalis* activity, with complete death in 12 h of incubation at minimum inhibitory concentration (MIC) of 100 μM. **3c** showed a dose-dependent cytotoxicity against mammalian VERO cell line, but the association of **3c** at 12.5 μM and metronidazole (MTZ) at 40 μM showed 95.31% activity against *T. vaginalis* trophozoites after 24 h of exposure and did not affect the VERO cell growth, appearing to be a good alternative. In silico analysis by molecular docking showed that **3c** could inhibit the activity of TvMGL (methionine gamma-lyase), TvLDH (lactate dehydrogenase), and TvPNP (purine nucleoside phosphorylase) affecting the *T. vaginalis* survival and also suggesting a different mechanism of action from MTZ. Therefore, these results propose that hydroxychalcones are promising anti-*T. vaginalis* agents and must be considered for further investigations regarding trichomoniasis treatment.

Keywords *T. vaginalis* · Synthesis · Chalcones · Molecular docking

Introduction

Trichomoniasis is the most common and prevalent non-viral sexually transmitted infection (STI) in the world. Based on estimates of the World Health Organization, trichomoniasis affects more than 276 million people every year worldwide (WHO 2012; Leitsch 2016). The treatment for human trichomoniasis is currently based on 5′-nitroimidazol agents, more specifically metronidazole (MTZ) and tinidazole (TNZ), which are the only drugs approved and recommended by the Food and Drug Administration (FDA-EUA n.d). However, this course of treatment has been showing some failures, which can be attributed to allergic reactions, side effects, and the emergence of resistant isolates to 5′-nitroimidazoles (Paulish-Miller et al. 2014). There are no alternative drugs approved for the treatment of such cases, establishing a dependence on a single therapeutic class, which is problematic. Thus, the search for alternative treatments to 5′-nitroimidazoles is necessary and has been frequently studied. In this context, synthetic compounds represent an important tool for the drug discovery (Bala and Chhonker 2018; de Brum Vieira et al. 2015).

In the discovery process for new antiparasitic drugs, the design and synthesis of novel compounds with high specificity

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is particularly important for the future development of clinically useful drugs. Thus, *Trichomonas vaginalis* proteins, such as lactate dehydrogenase (LDH), methionine gamma-lyase (MGL), and purine nucleoside phosphorylase (PNP), may serve as drug targets to predict potential inhibitors of *T. vaginalis*, because they differ from human proteins and are important for *T. vaginalis* survival (Setzer et al. 2017).

The use of the standard scaffold of chalcones (1,3-diphenyl-2-propen-1-one) enables a multiplicity of substitutions because of their easy synthesis, simple chemistry, and straightforward hydrogen atom manipulation, and such changes, e.g., addition of different chemical groups/radicals, can offer novel biological activities (Gomes et al. 2017; Wong 1968). Chalcones are α,β-unsaturated aromatic ketones reported as open-chain precursors for biosynthesis of flavonoids and isoflavonoids and widely distributed in nature, as part of foods and beverages. Its biological properties have been extensively studied, presenting promising activities such as antitumoral (Mahapatra et al. 2015), antifungal (Palanco et al. 2017), antioxidant, analgesic, anti-inflammatory (Abdellatif et al. 2015), antiparasitic (Borsari et al. 2017), and more importantly anti-*T. vaginalis* (Trein et al. 2019).

To indicate a new alternative for trichomoniasis treatment, we describe here the synthesis and antiparasitic activity of three chalcone analogues, more specifically hydroxychalcones, alone and in association with metronidazole. In addition, analyses of the biochemical effects against trophozoites of *T. vaginalis*, cytotoxicity against VERO cells, and molecular docking with *T. vaginalis* enzymes were performed to indicate a more in-depth profile on the behavior of these compounds.

Materials and methods

Chemistry: synthesis and identification of hydroxychalcones

All reagents and solvents employed in the synthesis of chalcones were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Reactions were monitored by thin-layer chromatography (TLC) using hexane/ethyl acetate and observed under ultraviolet light. Hydroxychalcones **3a**, **3b,** and **3c** were synthesized by Claisen-Schmidt condensation based on a previously described procedure (Fig. 1) (Wang et al. 2014). Briefly, an aqueous solution of sodium hydroxide 40% (2.0 mL) was added to a round bottom flask containing 15 mL of ethanol and 1.0 mmol of 2′-hydroxyacetophenone, in an ice bath. After 30 min, 1.2 mmol of the aromatic aldehyde was added and the mixture was stirred at room temperature until completion. Diluted hydrochloric acid was then used to neutralize the reaction mixture; the precipitate was filtered by gravity filtration and washed with cold water. The dried crude solid was purified by recrystallization with hot ethanol. Chalcones **3b** and **3c** were reported before by our group (Da Silva et al. 2018a, b), and products **3a–c** were confirmed by their melting points (Fisatom 430 apparatus), infrared spectroscopy (FTIR-Prestige 21), and mass spectrometry (GC-MS-QP 2010 SE Standard Gas Chromatograph-Mass Spectrometer with an AOC-20 automatic injector, RTx-5MS column, using helium as carrier gas). The data related to the identification of synthetic hydroxychalcones is displayed in Table 1.

VERO cell line culture

Mammalian VERO cell line, obtained from the Bank of Cells of Rio de Janeiro (BCRJ, University of Rio de Janeiro), was cultured in monolayer in RPMI 1640 medium (Vitrocell Embriolife, Campinas, Brazil), supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% L-glutamine, and 1% penicillin/streptomycin, and kept in a CO_2 incubator with humidified air (5% CO_2 at 37 °C).

Cytotoxicity assay against mammalian VERO cell line

To evaluate the cytotoxicity of hydroxychalcones, VERO cells were seeded in 96-well microtiter plates (Cral®) at a density of 2×10^4 cells per well in a volume of 100 μL and incubated at 37 °C in a humidified air atmosphere and 5% CO_2 for 24 h. When cells reached > 80% confluence, the cells were treated with hydroxychalcone, previously diluted in 0.6% dimethyl sulfoxide (DMSO), at concentrations varying from 12.5 to 200 μM and in association with MTZ. Untreated cells were used as negative controls. After 24 h of incubation in the CO_2 incubator with humidified air (5% CO_2 at 37 °C), viability of the cell lines was evaluated by the MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide method. Differences in viability after treatment were measured by spectrophotometry through a plate reader (Mindray) at 492 nm. All assays were performed independently at least three times in quadruplicate, and results were expressed as the percentage of cell growth inhibition in comparison with the negative control.

Trichomonas vaginalis culture

T. vaginalis 30236 isolate, obtained from American Type Culture Collection (ATCC), which is normally susceptible to metronidazole, was used in this study. Trophozoites were axenically cultured in vitro in a trypticase-yeast extract-maltose (TYM) medium without agar (pH 6.0), supplemented with 10% sterile heat-inactivated bovine serum and 5 mg/mL streptomycin, and then incubated at 37 °C (Diamond 1957).

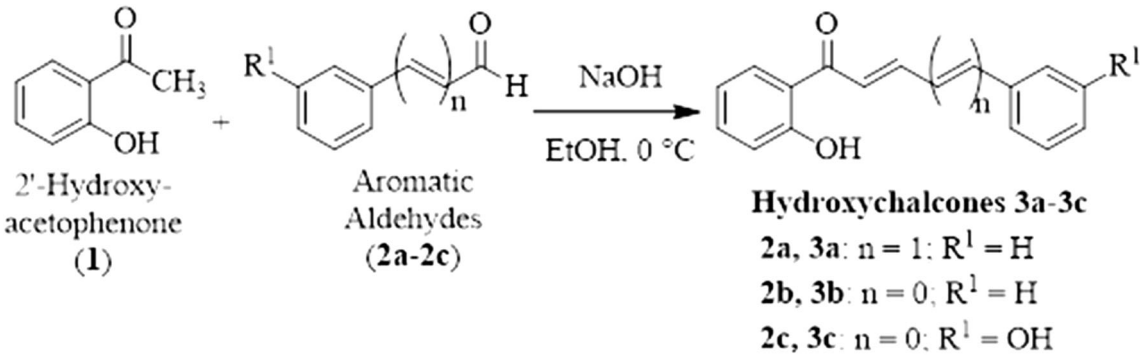
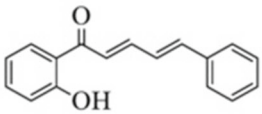
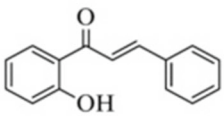
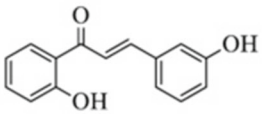


Fig. 1 Synthesis of hydroxychalcones **3a**–**3c**

Anti-*T. vaginalis* assays

Anti-*T. vaginalis* activity, minimum inhibitory concentration (MIC), association with metronidazole, and kinetic growth assays were performed following the methodology of Sena-Lopes et al. (2017). Initially, for the anti-*T. vaginalis* assays, cultures with viability equal or higher than 95% were used after being analyzed through observation of motility, morphology, and trypan blue dye exclusion (0.4%) assay under a light microscope at × 400 magnification. The activity of hydroxychalcones was screened in vitro against *T. vaginalis* in 96-well microtiter plates (Cral®). A volume

Table 1 Data on hydroxychalcones **3a**–**3c**

Code	Structure	Yield	CLog P[a]	Melting point (°C)	Exact Mass
3a (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one		60%	4.4	Exp.: 151 – 152 Lit.: 150 – 152 (Desideri et al., 2003)	250.10
3b (*E*)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one		46%	3.9	Exp.: 90 – 91 Lit.: 89 (Jhala et al., 2006)	224.08
3c (*E*)-1-(2-hydroxyphenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one		32%	3.3	Exp.: 163 – 165 Lit.: 165 – 167 (Kamboj et al., 2010)	240.08

[a] Theoretical value of CLog P determined by ChemDraw software 8.0, CambridgeSoft, Cambridge, MA

of 150 μL of trophozoite solution was seeded at an initial density of 2.6×10^5 trophozoites/mL and incubated with 50 μL of a solution containing TYM medium and hydroxychalcones at a final concentration of 100 μM, previously diluted in 0.6% DMSO. Three controls were applied in each assay: a negative control containing only trophozoites, a 0.6% DMSO control, and a positive control containing 100 μM of MTZ (Sigma-Aldrich, St. Louis, USA). The microtiter plates were incubated at 37 °C with 5% CO_2 for 24 h. After that, a preparation containing trophozoites and trypan blue (0.4%) at a ratio of 1:1 was counted in a Neubauer chamber, and trophozoite motility and morphology was analyzed by light microscopy while viability was assessed through trypan blue dye exclusion (0.4%) assay. Only the compounds that reduced the viability of parasites by 100% were used in the following experiments, from now on referred as active hydroxychalcone(s).

The MIC values against *T. vaginalis* were established in the same conditions as described above with variations on the concentrations of the active hydroxychalcone. After MIC determination, confirmation was performed by transferring the culture solution from MIC wells and from the concentrations directly below and above, as well as controls, to tubes containing fresh TYM medium which were then reincubated at 37 °C with 5% CO_2. Trophozoites were counted in a Neubauer chamber every 24 h during 96 h to confirm MIC, and the viability was assessed by trypan blue dye exclusion (0.4%) assay.

The effect of the association between the active hydroxychalcone and MTZ was evaluated following the methodology of Hübner et al. (2016), established in 96-well microtiter plates where trophozoites were treated considering the association of the following incubation conditions: 40 μM or 20 μM of MTZ + active hydroxychalcone at non-cytotoxic concentrations for the VERO cell line.

A kinetic growth curve was established to obtain a more accurate profile for hydroxychalcone activity against *T. vaginalis*. Trophozoite viability was observed by light microscopy after incubation at respective MIC or in association with MTZ. Growth analysis was performed at 1, 6, 12, 24, 48, 72, and 96 h by trypan blue dye exclusion (0.4%) assay.

For all assays, untreated parasites were used as negative controls. Plates were incubated at 37 °C with 5% CO_2 for 24 h. After that, trophozoite motility and morphology was analyzed by light microscopy counted in a Neubauer chamber, while viability was assessed through trypan blue dye exclusion (0.4%) assay. All assays were performed independently at least three times in triplicate, and results were expressed as the percentage of viable trophozoites in comparison with the negative control.

Molecular docking

The binding modes of active hydroxychalcone with *T. vaginalis* methionine gamma-lyase (TvMGL; PDB: 1E5E), lactate dehydrogenase (TvLDH; PDB 5A1T), and purine nucleoside phosphorylase (TvPNP; PDB 1Z36) were predicted using the software AutoDock Vina 1.1.2 (Trott and Olson 2009). The crystal structure of TvMGL, TvLDH, and TvPNP was retrieved from the Protein Data Bank (http://www.pdb.org/pdb/) and prepared by Chimera 1.5.3, including removal of ligands (Pettersen et al. 2004). A grid box size covering the residues in the active site of the proteins was implemented by AutoDock Tools 1.5.6 (Morris et al. 2009). The active hydroxychalcone was built and optimized in the software Avogadro 1.1.1. (Hanwell et al. 2012). Docking poses of the investigated compound were visualized using Accelrys Discovery Studio 3.5.

In silico drug-likeness evaluation

The active hydroxychalcone was studied by applying the Lipinski's rule of five, in which the criteria, established by Lipinski, to evaluate the theoretical oral bioavailability were as follows: partition coefficient (miLogP) ≤ 5.0, molecular weight ≤ 500, hydrogen bond acceptors ≤ 10, and donors ≤ 5. The compounds that violate one or more rules demonstrate an unattractive oral bioavailability. In addition, physicochemical property analyses of active hydroxychalcone were performed on software ADMET Predictor with parameters of 1 mg/kg of 3c administered orally in humans (Lipinski 2004).

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) using a probability value of $p < 0.05$ using the GraphPad Prism 8.2.0 software. For screening and MIC assays, Tukey's post-test was conducted to allow multiple comparisons between all treatments. For association with metronidazole and cytotoxicity assay, Dunnett's post-test was conducted to identify significant differences between the negative control and the means of different treatments. For the kinetic growth curve, two-way ANOVA was conducted followed by Bonferroni post-tests. The IC_{50} and CC_{50} values were assessed through a non-linear regression model.

Results

Synthesis and identification of hydroxychalcones

Regarding the synthesis of the hydroxychalcones, it can be seen in Table 1 that three products were obtained in moderate yields (32–60%). Infrared and mass spectra of products **3a**,

3b, and **3c** are displayed in Figs. 2 and 3 and were in agreement with the proposed structures. Infrared spectra showed absorption bands related to the carbonyl (1635 cm^{-1}) and hydroxyl groups (3300 cm^{-1}). The molecular ions were identified for all molecules, matching their exact mass, as demonstrated in the mass spectra, indicating that the target products were obtained satisfactorily.

Cytotoxicity assay against mammalian VERO cell line

The MTT assay showed that **3c** decreased cell viability in a dose-dependent manner. The CC_{50} value was established as 118.9 μM, and a significant cell growth inhibition was observed in VERO cells after treatment with **3c** at MIC concentration, which inhibited 47.7% of growth. However, from the concentration of 25 μM and below, there was no inhibition of cell growth. Thus, the association of **3c** at 12.5 μM and MTZ at 40 μM did not inhibit cell growth after 24 h of exposure, showing no significant difference when compared with the negative control (NC) (Fig. 4).

Anti-*T. vaginalis* assays

Analysis of the data obtained in the evaluation of anti-*T. vaginalis* assay showed that hydroxychalcones **3a**, **3b,** and **3c** at 100 μM, after 24 h of exposure, induced 12%, 54%, and 95% of trophozoite death, respectively, showing the potential antiparasitic activity of all three compounds. Nonetheless, at tested concentration, **3c** was the most potent without showing a significant statistical difference from the positive control (MTZ at 100 μM), as indicated by letter "b" in Fig. 5A. As expected, the positive control was stained with trypan blue (0.4%) and exhibited negative motility, reducing 100% viability of trophozoites. In addition, the control group DMSO (0.6%) showed positive motility, did not stain with trypan blue (0.4%), and did not present significant statistical

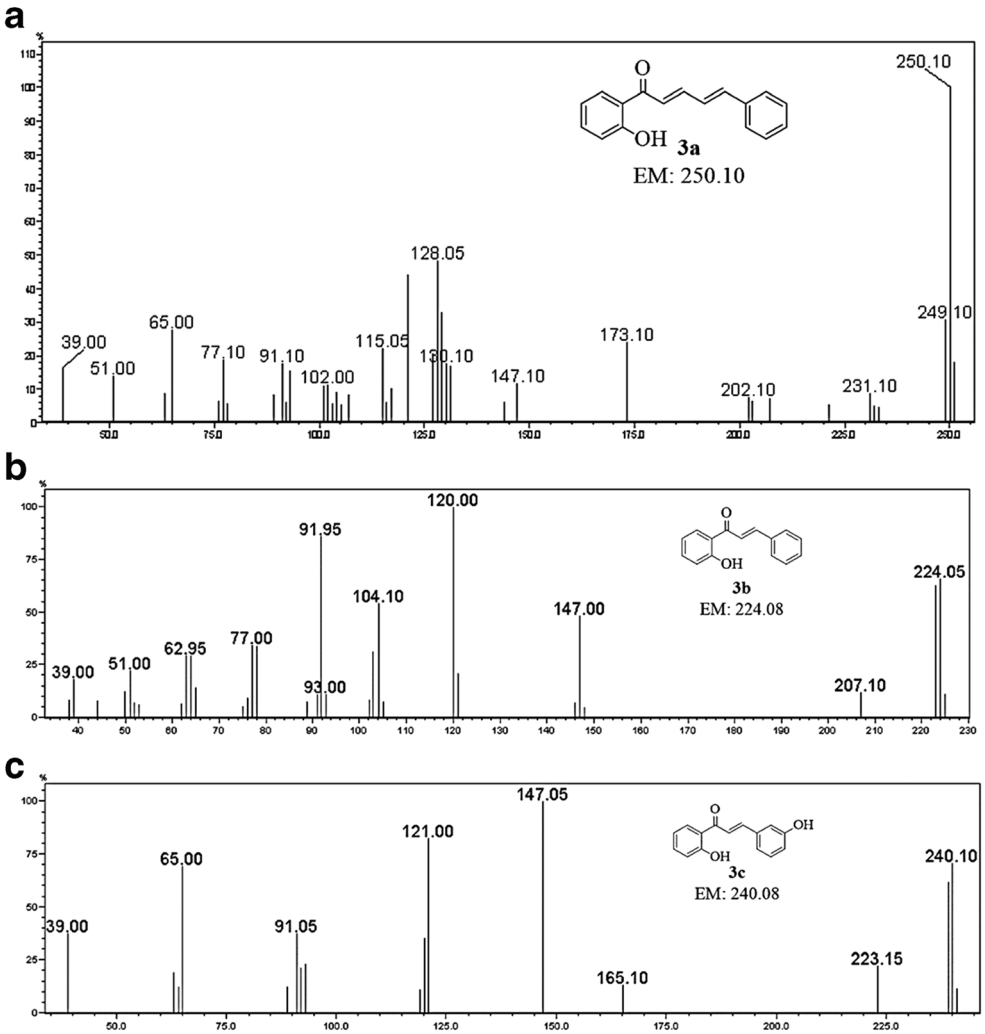


Fig. 2 Mass spectrum of hydroxychalcones **3a** (A), **3b** (B), and **3c** (C)

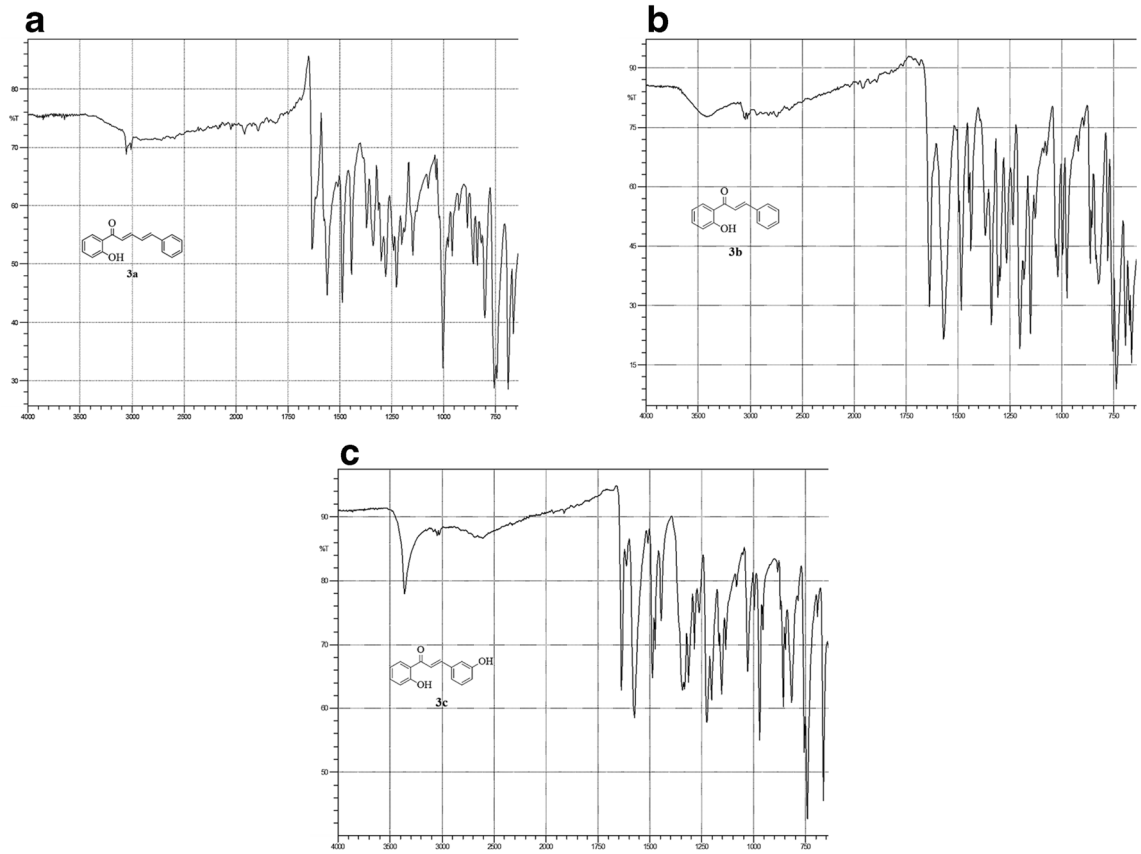


Fig. 3 Infrared spectrum of hydroxychalcones **3a** (A), **3b** (B), and **3c** (C)

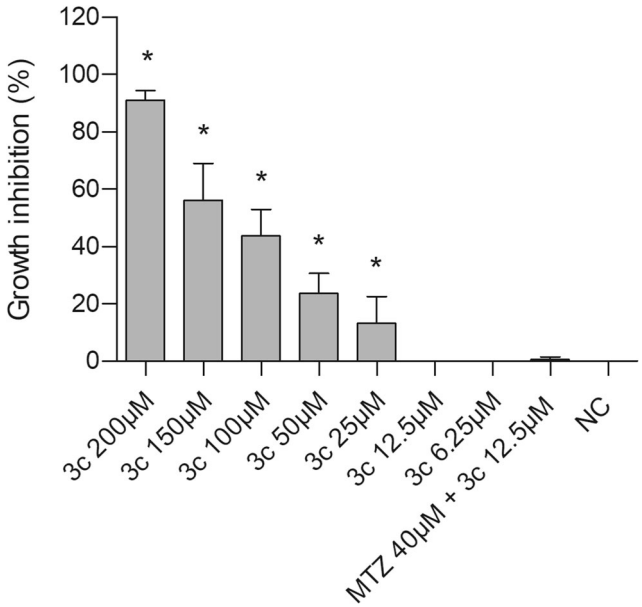


Fig. 4 Cytotoxicity effect of hydroxychalcone **3c** alone at 6.25–200 μM and of **3c** (12.5 μM) in association with MTZ (40 μM) against VERO cell line. Cell proliferation was investigated by MTT assay. Data are expressed as means ± SD from three independent experiments, analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. (*) represents a significant difference when compared with the negative control (NC). For all $p < 0.05$

difference from the negative control (untreated trophozoites), as indicated by letter "a" (Fig. 5A).

The IC_{50} was established as 50.64 μM, and as a result of MIC assay, the ideal concentration for complete trophozoite death was established as 100 μM, which showed a significant difference from the positive control (MTZ at 100 μM) as indicated by letter "b" (Fig. 5B). The effect of **3c** association with MTZ against *T. vaginalis* trophozoites showed that hydroxychalcone **3c** at 12.5 μM in association with MTZ at 40 μM reduced the trophozoite viability by 98% after 24 h of incubation (Fig. 5C). The kinetic growth curve showed that time of exposure affect trophozoite growth after treatment with **3c** alone and in association with MTZ. **3c** alone at MIC concentration was able to reduce the trophozoite growth by 86%, and **3c** at 12.5 μM in association with MTZ at 40 μM reduced trophozoite growth by 89.2%, both at 12 h of exposure (5D) with complete death characterized at 24 h exposure (Fig. 5A, C).

Molecular docking

As depicted in Fig. 6A, B, the binding mode of the compound **3c** with the active site of TvMGL involves conventional hydrogen bonds with THR114 (2.65 Å) and LYS209 (3.14 Å). In addition, pi-donor hydrogen bonds, pi-alkyl, pi-anion, and van

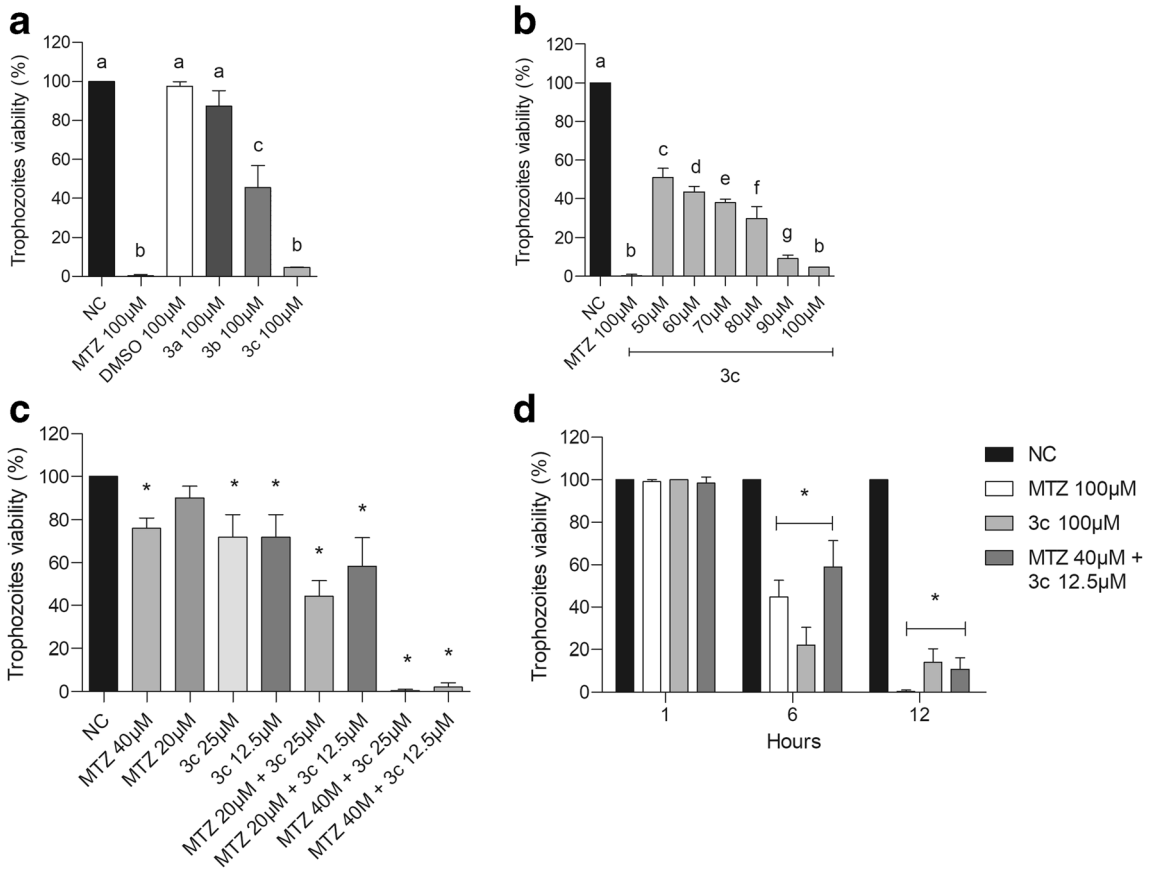


Fig. 5 (A) Anti-*T. vaginalis* activity of hydroxychalcones **3a**, **3b**, and **3c** at 100 μM confirmed by trypan blue dye exclusion assay (0.4%) after 24 h of exposure. (B) Determination of **3c** MIC after *T. vaginalis* 30236 isolate treatment at 50, 60, 70, 80, 90, and 100 μM after 24 h of exposure. (C) Anti-*T. vaginalis* effects of associations of different concentrations of **3c** and MTZ. (D) Kinetic growth curve of *T. vaginalis* 30236 isolate after treatment with **3c** alone and in association with MTZ at the period of 1, 6, 12, 24, 48, 72, and 96 h. Vehicle for solubilization at 0.6% (DMSO), metronidazole at 100 μM (MTZ), negative control (NC). Data are expressed as means ± SD from three independent experiments: (A, B) analyzed by one-way ANOVA followed by Tukey's multiple comparison test, (C) analyzed by one-way ANOVA followed by Dunnett's multiple comparison test, and (D) analyzed by two-way ANOVA followed by Bonferroni post-tests. Different letters (a–g) indicate a significant difference between treatments, and (*) represents a significant difference when compared with the negative control (NC). For all $p < 0.05$

der Waals forces contribute to the binding mode of compound **3c** with TvMGL, yielding binding free energy (ΔG_{bind}) of − 7.8 kcal/mol. A likely binding mode of the compound **3c** in the active site of TvLDH is depicted in Fig. 6C, D (ΔG_{bind} of − 7.4 kcal/mol). A hydrogen bond is formed between compound **3c** and THR239 residue (2.40 Å), while non-covalent interactions with other amino acid residues help to maintain the binding mode of the compound **3c** with TvLDH. The best mode of compound **3c** with TvPNP is represented in Fig. 6E, F, with a docking score of − 7.8 kcal/mol. ASP204 (2.40 Å) is involved in a hydrogen bond with compound **3c**, while pi-alkyl and pi-sigma interactions and van der Waals forces are formed between **3c** and other amino acid residues in the active site of TvPNP. In addition, the **3c** physicochemical properties showed that **3c** does not violate any of the 5 Lipinski rules, meaning it could have favorable properties for administration in humans and may be used as candidate to the study and development of a new antiparasitic agent (Table 2).

Discussion

The treatment of trichomoniasis relies on a single class of drugs, the 5′-nitroimidazoles, which are the most prescribed and effective drug against *T. vaginalis*. But, the development of resistant *T. vaginalis* isolates across the globe has made the research and development of alternatives to metronidazole treatment extremely necessary (Bala and Chhonker 2018).

In the development of new drugs, chalcones and chalcone derivatives possess a large number of different biological activities, which are highly appreciated in many areas, because they are bioactive against virtually all eukaryotes and some prokaryotes, and their molecular targets are numerous (Zhou 2015). During the last decades, numerous investigations have been carried out on the pharmacological activities of natural and synthetic chalcones. Some studies show that chalcones tested against *T. vaginalis* are usually associated with other chemical class, such as a study by Anthwal et al. (2014) that

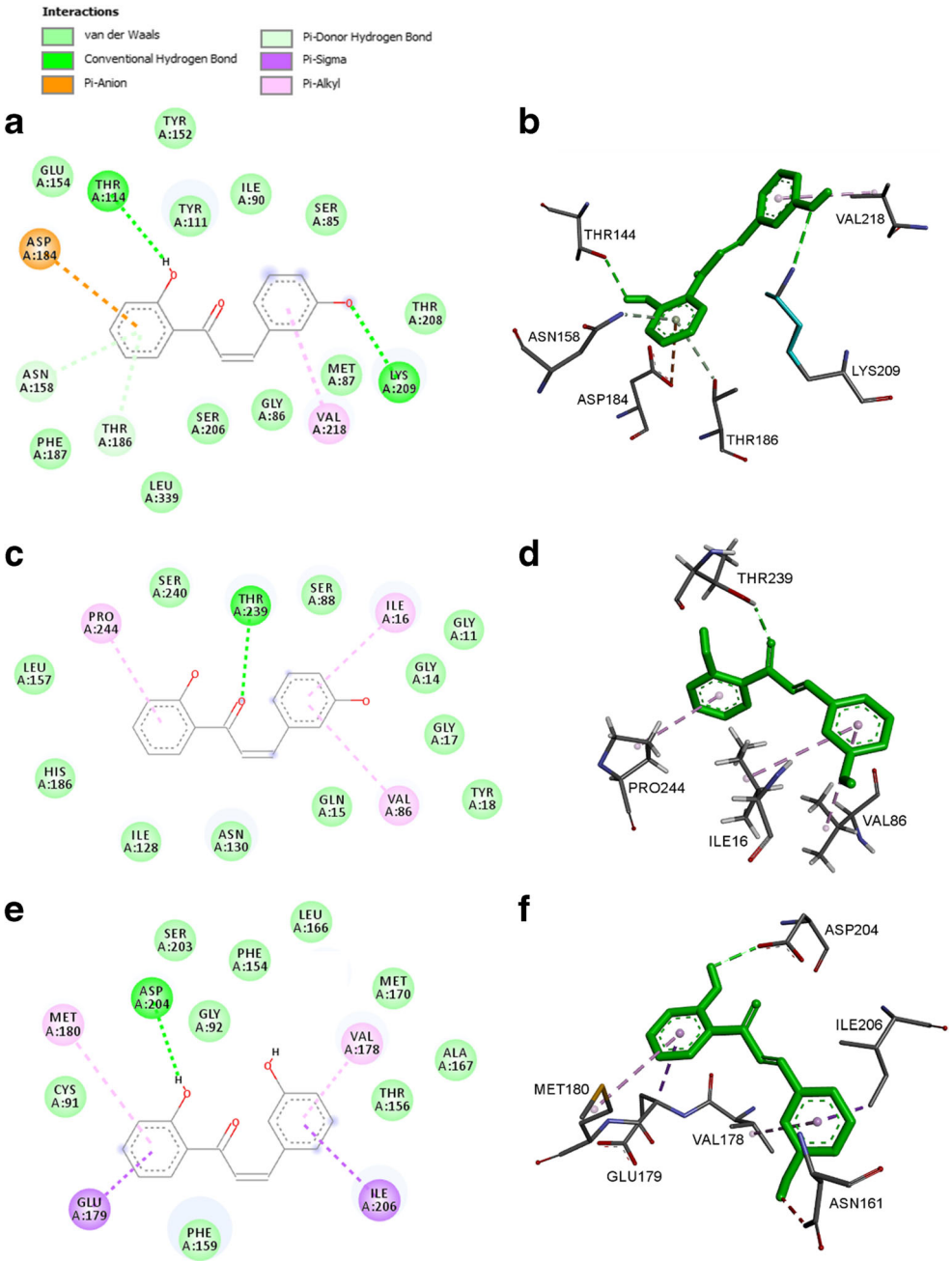


Fig. 6 Predicted binding mode of compound **3c** in the active site of TvMGL (A and B), TvLDH (C and D), and TvPNP (E and F)

shows that metronidazole-chalcone conjugates exhibited anti-*T. vaginalis* activity against MTZ-susceptible and MTZ-resistant *T. vaginalis* strains and another by Singh et al. (2016) that described a series of chalconyl blended triazole allied silatranes displaying significant activity against *T. vaginalis*.

Another alternative is the addition of substituents in aromatic rings, developing chalcone derivatives that might present different biological activities. In this manner, a recent study showed the potential activity of 3′-aminochalcone against *T. vaginalis* (Trein et al. 2019), and here we present the potential activity of another type of chalcone derivate, the hydroxychalcones. Until now, there is no data reported about hydroxychalcones with activity against *T. vaginalis* trophozoites; however, Borsari et al. (2017) discovered methoxylated 2′-hydroxychalcones as potent anti-*Trypanosoma brucei* agents, showing antiprotozoal activity. Thus, in this study, we evaluate the use of three hydroxychalcones for in vitro potential against *T. vaginalis*.

All synthetic products tested in this study are chalcones containing hydroxyl groups in their structure; specifically, all of them possess a hydroxyl group at the *–ortho* position of ring A (derived from the reagent 2′-hydroxyacetophenone). The different results determined in the anti-*T. vaginalis* assay

Table 2 Physicochemical properties of hydroxychalcone **3c** analyzed by the software ADMET Predictor. The data are based on a dose of 1 mg/kg administered orally in humans

Hydroxychalcone 3c—physicochemical properties—1 mg/kg in humans	
Lipinski's rule of 5	Do not violate
Percent unbound to blood plasma proteins in humans	4.609%
Volume of distribution in humans in steady state	0.631 L/kg
Blood-brain barrier penetration in humans	Low (40%)
Effective human jejunal permeability	7.363 cm/s × 10^4
Water solubility	0.084 mg/mL
Octanol-water partition coefficient (Log P)—lipophilicity	3.51
Predicted clearance (metabolism, renal, hepatic uptake)	Metabolism
Clearance by CYP	CYP1A2, CYP2C9, CYP2E1
Fraction absorbed in 24 h in humans	99.99%
Fraction bioavailable in 24 h in humans	92.33%
Maximum plasma concentration reached in 24 h in humans	16.61 mg/mL
Time at which maximum plasma concentration is reached in humans	1.8 h

demonstrated that although being from the same subclass of compounds, the structural differences influenced their antiparasitic potential. Hydroxychalcone **3a** is derived from cinnamaldehyde, which itself has been reported to exhibit anthelmintic activity against *Dactylogyrus intermedius* (Ling et al. 2015) and, because of that, has a central 5-carbon linker and an additional unsaturation when compared with **3b** and **3c**. However, product **3a** did not exert anti-*T. vaginalis* activity at the tested concentration so that the additional unsaturation does not seem to have an effect against this strain, or it possibly stabilizes the hydroxyl group in a way that prevents an inhibitory interaction with *T. vaginalis* cells. This is, nonetheless, an interesting result because, as shown by Rezk et al. (2002), a certain biological activity can differ significantly for a starting reagent and its derived synthetic product.

Hydroxychalcones **3b** and **3c** have a 3-carbon linker, but **3c** has another hydroxyl group at the *–meta* position of ring B (derived from the 3-hydroxybenzaldehyde). Borsari et al. (2017) investigated the antiparasitic potential of chalcones from the same class reported herein (2′-hydroxychalcones) but containing also methoxy substituents in the aromatic rings. Most of the methoxylated 2′-hydroxychalcones with an antiparasitic activity against *Trypanosoma brucei* were also substituted in the *–meta* position of ring B, which could indicate an important pattern of substitution associated with a pharmacological potential. It seems that the additional hydroxyl substituent present at chalcone **3c** contributed for its higher potential, while the length of the conjugated bonds (i.e., **3a**) did not seem to play a crucial role for the antiparasitic activity. Other synthetic hydroxychalcones with different substitution patterns can be obtained for a better understanding of the structural motifs required for an anti-*T. vaginalis* activity.

To evaluate the cytotoxicity of hydroxychalcone **3c**, we used mammalian VERO cell line. After 24 h of exposure, the CC_{50} value was established as 118.9 μM and **3c** at MIC concentration showed a cell growth inhibition of 47.7%, which is significantly high. No other previous report was found with the evaluation of hydroxychalcone **3c** in VERO cells, although two research articles performed toxicity assessments of molecules with the same structures of **3b** and **3c**. Lee et al. (2014) investigated the in vivo toxicities of some synthetic chalcones in zebrafish embryos, in which compounds equal to **3b** and **3c** (original codes: **1a** and **1d**, respectively) did not show significant differences from the untreated control in concentrations of up to 5 ppm (≈ 20 μM). At a concentration of 3 ppm of **3c** (≈ 12.5 μM considering its molar weight of 240.25), the survival rate was kept near 100% for 60 h post-fertilization. These results are in agreement with our in vitro assays, as the cell viability starts to decrease at concentrations above 25 μM. Forejtníková et al. (2005) demonstrated that hydroxychalcones, including **3b** and **3c**, display in vitro chemoprotective and toxic potentials depending on the tested concentration. In their report, chalcone equal to **3c** (original code: chalcone 2′,3-diOH; Table 1) inhibited 10% of CYP1A-dependent EROD activity and its IC_{50} cytotoxicity in rat liver epithelial WB-F344 cells was higher than 50 μM.

Considering **3c** cytotoxicity on VERO cells, the pharmacological effects of chalcone **3c** are dose-dependent and could be optimized to avoid adverse effects. Other synthetic approaches could be attempted to enhance the antiparasitic activity of chalcone **3c** and decrease its cytotoxicity effect, such as hydrogenation of its double bond to form a dihydrochalcone (Forejtníková et al. 2005), or cyclization of **3c** to yield a chromenone (Badavath et al. 2016). Other

possibility is the cytotoxicity evaluation in other cell lines, to get a better understanding of this cytotoxic effect, and alternatives such as nanoencapsulation and topical application have many advantages for drug delivery, because they can increase compound's interaction with tissues and cells, bioavailability, and drug targeting consequently resulting in increased efficacy and decreased drug adverse effects (Bouchemal et al. 2017; Frank et al. 2015). Thereby, these approaches can allow the use of lower concentrations of treatment.

Due to the limited treatment options for trichomoniasis, the association of different chemical classes is a good alternative and allows the use of lower doses of the constituents, a situation that may reduce adverse reactions and overcome the resistant strain problem (Tallarida 2011). To confirm if the use of lower concentrations of **3c** could be applied in the treatment of trichomoniasis, we chose to test the effect of **3c** in association with MTZ in different concentrations determined from **3c** MIC value (100 μM) and MTZ MIC value (80 μM) against *T. vaginalis* ATCC 30236 trophozoites, assessed under laboratory conditions (data not shown).

The best association was the use of **3c** at 12.5 μM and MTZ at 40 μM that reduced trophozoite viability by 89.2% in 12 h of exposure and led to complete death in 24 h, similar to anti-*T. vaginalis* assay results for **3c** alone at MIC concentration. The results demonstrate that the interaction between both compounds reduces the concentration required to cause parasite death. Since **3c** concentration of 12.5 μM did not affect the growth of mammalian VERO cells, this is a good alternative to reduce the possible side effects caused by cytotoxicity. In addition, this association also allows the use of a lower concentration of metronidazole. In cases of resistance to this drug, it is necessary to increase the treatment concentration, increasing the possibility of adverse effects such as headache, nausea, diarrhea, and a metallic taste, and consequently decrease or impair treatment adhesion by the patient (Kissinger 2015).

Molecular docking analyses with three *T. vaginalis* enzymes were performed to investigate the possible molecular mechanism responsible for the anti-*T vaginalis* activity of compound **3c**. TvMGL is a pyridoxal-5-phosphate-dependent enzyme that catalyzes an α,γ-elimination reaction on L-methionine to generate ammonia, methanethiol, and α-oxobutyrate (Lockwood and Coombs 1991). Since mammals do not have MGL, developing drugs targeting this enzyme is a rational strategy to reduce the toxicity following anti-*T vaginalis* treatment. TvLDH plays an important role in *T. vaginalis* metabolism via generation of NAD+ required for glycolysis through the reduction of pyruvate to lactate (Steindel et al. 2016). TvPNP is important for *T. vaginalis* survival, since these protozoa lack de novo synthesis of purine nucleosides, relying on the functions of TvPNP and nucleoside kinase (Rinaldo-Matthis et al. 2007). This metabolic feature makes the purine salvage pathway a key target for antiparasitic chemotherapy, reinforcing the promising anti-*T. vaginalis* action of **3c**.

The binding mode of compound **3c** with the active site of TvMGL, TvLDH, and TvPNP shows that **3c** could inhibit the active sites of these enzymes, affecting *T. vaginalis* survival as evidenced in our in vitro studies. Nonetheless, the activity of these enzymes in the presence of compound **3c** should be further investigated. The molecular docking studies are in agreement with the preliminary anti-*T. vaginalis* screening as they indicate that the additional *meta*-hydroxyl substituent of chalcone **3c** is an important binding site for TvMGL and TvLDH. As discussed before, carbonyl molecules containing a hydroxyl group at the –*ortho* position of the ring may form intramolecular bonding, which would prevent these groups from forming the hydrogen bonds (Da Silva et al. 2018a, b; Lee et al. 2014). Therefore, for hydroxychalcone **3c**, even if that happened, the *meta*-hydroxy group would still be available for interaction with the evaluated enzymes, which would not be possible for chalcones **3a** and **3b**. Also, when a hydroxyl group loses its hydrogen, it forms a phenoxy radical with unpaired electrons which could be responsible for the predicted alkyl bonding.

Since MTZ did not present a good binding mode with the tested enzymes (data not shown), we believe that the association of different mechanisms of action of compounds **3c** and MTZ is responsible for the parasites' death. Whereas MTZ mechanism of action is based on drug penetration into the cell wall of *T. vaginalis* by passive diffusion, activation in the hydrogenosomes, anaerobic reduction of the nitro group by the enzyme pyruvate ferredoxin oxidoreductase (PFOR) forming nitro cytotoxic radicals, inhibition of the synthesis and degradation of DNA strands, and consequently the death of trophozoites (Petrin et al. 1998), here we suggest that **3c** mechanism of action may be the inhibition of three important enzymes for *T. vaginalis* survival.

An effective drug must reach its specific target in sufficient concentration since early stages, such as biologic screenings for identification of a potential molecule (Gajdács 2019). Thus, computer models constitute valid alternatives to perform assessments of absorption, distribution, metabolism, and excretion (ADME) increasingly earlier in the drug development process (Daina et al. 2017). In this work, the drug-likeness was established from structural or physicochemical inspections. The hydroxychalcone **3c** showed properties probably compatible with an acceptable pharmacokinetic profile, such as being available for oral administration, being absorbed, and being bioavailable in 24 h. Besides that, the results of the interaction between **3c** with cytochromes P450 (CYP), especially with the isoforms CYP1A2, CYP2C9, CYP2E1, allow to infer the occurrence of metabolic biotransformation and consequently lower probability of adverse effects, thus demonstrating the pharmacological potential of these compounds for the development of a new anti-*T. vaginalis* drug.

Conclusion

Hydroxychalcone **3c** demonstrated a potent anti-*T. vaginalis* activity against ATCC 30236 isolate, and the association of **3c** and MTZ in lower concentrations also displayed a good anti-*T. vaginalis* activity, not affecting VERO cell growth, which indicates that this association may not cause damage to human cells and can be considered a new alternative treatment for trichomoniasis. In addition, enzymes related to *T. vaginalis* survival could be inhibited by **3c** binding, suggesting a molecular mechanism of action different to MTZ, which allows lower doses of both compounds, reducing possible adverse effects caused by high cytotoxicity, and can also prevent cross-resistance development. Together, these results indicate that hydroxychalcones can be considered a good option for development of a new alternative drug for trichomoniasis treatment in association with metronidazole.

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